Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

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LONG-TERM GOALS

Quantifying physiological indicators of stress in wild marine mammals and the interrelationships between different stress markers can be used to estimate the impact of anthropogenic stressors on marine mammal populations. The United States Navy, as part of its environmental stewardship, can utilize stress markers to assess the acute and chronic impacts that its actions might have on marine mammals. This approach would permit better mitigation of potential impacts and ensure that Navy activities do not come at a deleterious cost to marine mammal populations.

OBJECTIVES

The objectives of this effort are to: 1) determine the variation in corticosteroid hormones, thyroid hormones, and catecholamines within a dolphin population relative to seasonality, time of day, gender, age and reproductive state; 2) assess relationships between serum corticosteroid levels and levels found in other matrices (i.e. biological samples), including feces and blubber; 3) and to perform stress tests and thyroid stimulating hormone (TSH) challenges to characterize the activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axes across multiple matrices, respectively.

APPROACH

Task 1 – Seasonal variations in hormones across multiple matrices
Regular sampling from different matrices (e.g. blubber, blood, feces) will be collected from the U.S. Navy Marine Mammal Program (MMP) dolphin population over the course of a year. Subject dolphins will be split into categories based upon age and will be sampled bi-weekly throughout the year for blood and feces. A subset of animals will be selected for monthly blubber biopsies. Blood and fecal samples will be collected from dolphins through their voluntary participation. Blood collections will be made from the ventral fluke from the arteriovenous plexus and collections will be made between 0700-1000. Fecal samples will be collected by use of a suction catheter inserted into the anal orifice of the dolphin the day after the blood collection. Bimonthly blubber biopsies will be collected with a 16g tissue biopsy needle.

Serum samples will be processed for adrenocorticosteroids, catecholamines, and thyroid hormones via radioimmunoassay (RIA). Parallel processing of serum catecholamines will be performed via high-performance liquid chromatography (HPLC) to assess variability in the measurement methods. Metabolites of cortisol, aldosterone and thyroid hormone will be extracted from fecal samples and measured via RIA using established techniques (Wasser et al., 2010). A multi-step biphasic organic solvent extraction will be used to isolate the corticosteroids from the blubber tissue (Kellar et al., 2009). The hormones will be measured using a commercially available enzyme immunoassay (EIA) and parallel processing via HPLC will be used to verify method performance.

Task 2 – Diurnal variation in hormone production
Hormones will be assessed for diurnal variation during the second year of the study. Ten dolphins will be selected for repeat testing throughout the year. Blood samples will be collected from the dolphins at monthly intervals via voluntary venipuncture of the arteriovenous plexus on the ventral fluke. Samples will be collected first thing in the morning (~0700), at noon, and in the late afternoon (~1700). Blood samples will be processed via RIA and HPLC as described under Task 1. Similar analyses will be conducted on serially collected scat of these 10 individuals over the same 24 hr period and a second 24 hr period one week later when not being sampled for blood.

Task 3 – Adrenocortical sensitivity
Adrenocortical sensitivity and the relationship between corticosteroids in serum and other matrices will be determined by submitting five dolphins to an out-of-water stress test. Dolphins will be beached and blood samples and blubber biopsies collected over a two-hour period. Blood samples will be collected for an additional two hour period once the dolphin is placed back in the water. Blood and fecal samples will be collected two days prior and two days following the procedure to compare changes vs. baseline and to characterize the recovery period. (Note* The original study design for assessing adrenocortical

sensitivity involved the administration of ACTH slow-release gel. Pilot results indicated that the gel did not reliably induce an adrenocortical response and the study design was modified accordingly.) An additional five dolphins will be fed fish containing cortisol pellets (60 mg every 6 hours) in order to elevate serum cortisol over a period of five days. Voluntary blood and fecal samples will be collected daily to determine if serum cortisol levels are elevated and sustained. Procedures will be coupled to blubber biopsies so cortisol deposition in the blubber can be assessed.

Task 4 – Thyroid challenges
Five dolphins will be given a TSH stimulation test. A pre-test blood draw will be collected from each dolphin while it is in its enclosure. The dolphin will then be removed from the water to a location suitable for the procedure. A bolus injection of TSH, the dosage of which will be determined in a pilot study, will be intramuscularly administered. Blood samples will then be collected every 15 minutes for a period of one hour. Following completion of the sampling period, the dolphin will be returned to its enclosure and voluntary blood samples collected two and four hours following the injection. Blood samples will also be collected daily for three days following the injection. Baseline fecal samples will be collected prior to the first injection and will be collected daily for three days following the injection. (Note* This protocol was modified from the original in response to a recent publication of a TSH challenge in a single dolphin.)

WORK COMPLETED

Task 1 – Seasonal variation in stress hormones
A group of 30 bottlenose dolphins was identified from within the MMP population that could provide voluntary biweekly blood and fecal samples over a period of a year. The following distribution of animals was obtained:

Age (yrs)	Male	Female
5-15	6	4
16-25	3	3
25+	7	7

Monthly blubber biopsies were collected on the same day of the blood collections in four dolphins. Blubber biopsies were collected approximately 12-14 cm below the posterior insertion of the dorsal fins using a 16g biopsy needle. Two to three biopsies were taken each sample period to ensure that sufficient blubber was obtained for analysis.

A total of 735 blood collections were made out of a total of 778 possible draws (~94% success rate). A total of 638 matched fecal samples were collected such that 87% of the blood samples had matched fecal comparisons, and a total of 47 blubber biopsies were collected over the course of the study. Sample collection on this task is complete and all blood, fecal and blubber samples have been analyzed.

Task 2 – A group of 10 dolphins was identified to participate in the diurnal variation study. A total of 357 out of 360 possible blood draws were completed (99%) and a total of 215 out of a possible 240 fecal samples were collected (90%). All blood and fecal samples have been analyzed, except for serum aldosterone (to be processed under the extension grant described in *RELATED PROJECTS*).

Task 3 – All cortisol feeding trials are complete. Daily voluntary blood and fecal samples were collected and blubber biopsies were taken prior to cortisol feeding (day 0) and on the third and fifth day of feeding. All blood, fecal and blubber samples have been processed.

Pilot studies with ACTH administration were completed in the winter and spring of 2013/2014. The protocol was changed in the spring and stress tests were performed throughout the summer with completion in August 2014. All stress tests are now complete. Blood samples from the study were processed for all hormones, except epineprhine and norepinephrine (to be processed under the extension grant described in *RELATED PROJECTS*). All fecal and blubber samples have been processed.

RESULTS

Dolphins at the MMP produce low levels of corticosteroids but do not suffer from adrenal exhaustion or insufficiency. In many instances, levels are sufficiently low that alternative means of processing are being employed to accurately assess circulating levels. The low circulating levels likely reflect the lack of predatory and foraging stressors as well as disease mitigation. An incidental finding of megesterol acetate (MegAce) administration impeding cortisol production was found in the MMP population. Due to the use of megesterol acetate for controlling rut-behavior in male dolphins, this incidental finding has significant ramifications for the welfare of dolphins under human care.

Significant seasonal differences were noted in free and bound T3. Interactions between seasonality, age, and gender significantly affected all other hormones measured, e.g. ACTH was significantly higher in the spring than other times of the year, but only in males. Cortisol levels were highest in the oldest males (>27 years) and cortisol was signficantly higher in the morning in both sexes than it was in either the evening or at noon.

Determination of the biological half-life of cortisol and aldosterone permits levels observed in wild and captive animals to be placed in better context of collection methods (e.g. impact of handling) and provides insight on the kinteics of this corticosteroid. The biological half-life of cortisol calculated from hydrocortisone feeding study was found to be ~109 minutes; however, the half-life when calculated when cortisol was endogenously produced following the stress test was found to be 62.3 minutes (Figure 1). The biological half-life of aldosterone was determined to 28.7 minutes when relying only on the clearance of endogenously derived aldosterone. An acute stressor produced marked increases in cortisol and aldosterone to levels in excess of those commonly observed in wild-caught animals and comparable to or greater than prior ACTH stimulations (Thomson and Geraci, 1986; Figure 2). Cortisol and aldosterone varied directly with increasing ACTH and the magnitude of the change in aldosteone indicates that ACTH is a stronger secretagogue for aldosterone than cortisol. Elevations in cortisol were associated with suppression of ACTH secretion and free T3 levels, suggesting an active feedback control mechanism.

Under a natural and acute stressor, fecal cortisol closely follows serum cortisol levels over the course of hours, which is more rapid than observed in other mammalian systems. In this regard, fecal cortisol may provide a better indicator of the state of stress in a wild-caught dolphin that must experience handling stress prior to the collection of blood samples, i.e. it will reflect the state of the animal several hours prior to capture. However, under chronic elevations of cortisol, fecal glucocorticoid levels were reduced. The reduced appearance of fecal glucocorticoids suggests a possible change in cortisol

degrdation and clearnace (e.g. through changing CBG binding capacity), possible reabsorption from the gut, or some mechanism as of yet undefined.

Cortisol incorporation into blubber increased when cortisol was chronically elevated (~5 days). The time course for incorporation, as observed during the acute stress test, was dependent upon the duration of the applied stressor and was highly variable across individuals, probably due to individual differences in peripheral vascular dynamics. Thus, blubber cortisol seems like a feasible matrix for assessing cortisol in cetaceans, but across the duration of shorter-term stressors, the incorporation may be highly variable across individuals.

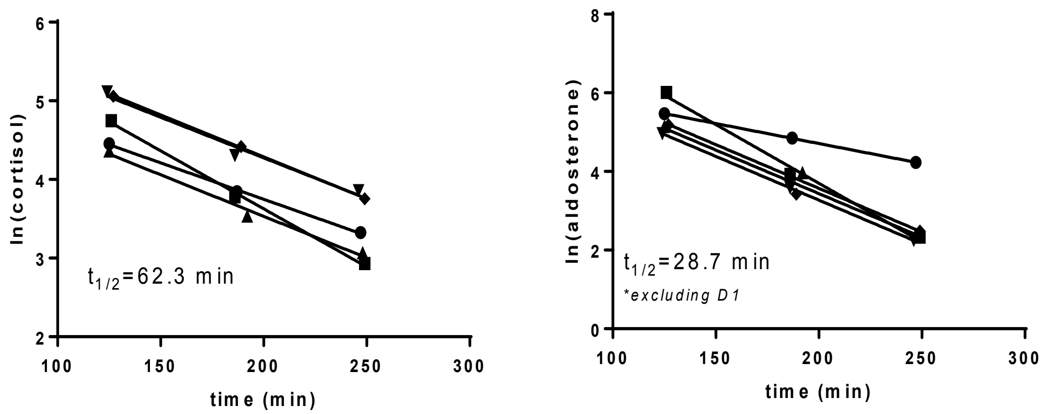

Figure 1. Panels showing the clearance of endogenously produced cortisol and aldosterone. The clearances were observed after elevating the hormones through application of an external stressor. The half-life of cortisol is 62.3 minutes. The half-life of aldosterone is 28.7 minutes.

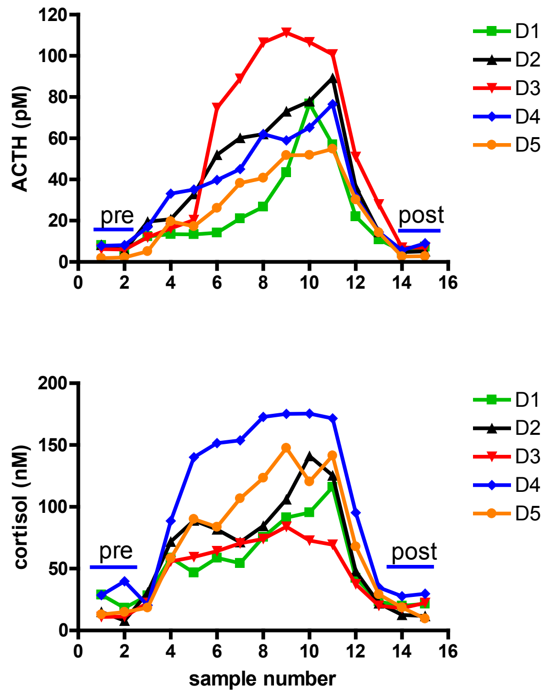

Figure 2. Change in ACTH and cortisol following a stress test in five bottlenose dolphins. The pre- and post- periods are values from blood samples collected the days prior to and following the test (samples 1 and 2, and samples 14 and 15, respectively). ACTH and cortisol rise rapidly after the stress test begins (sample 3), and decline rapidly once the test is concluded and the dolphin is returned to the water (samples 12 and 13, which are one and two hours after the test is concluded). The greatest changes in ACTH did not necessarily correlate with the greatest changes in cortisol.

The use of TSH to stimulate thyroid production met with limited success. The amount of TSH provided was ~67% greater than that used in a prior, recently reported TSH challenge (West et al. 2014). Dosages produced responses in circulating levels of T3 (free and total) in four out of five dolphins; the oldest, female dolphin showed no response to the TSH stimulation. At 24-hours after the injection, increases in free T3 ranged from 25.3-86.1% of baseline levels in responsive dolphins. Free and bound T4 were more consistent in their response to the TSH stimulation across all animals and increases in free and bound T4 24 hours after the stimulation ranged from 10.2-78.8% and 10.7-37.9% of baseline, respectively. Free T3 was signficantly and positively related to total T3 ($p<0.05$, $R^2=0.12$) but the relationship was weaker than that between free and total T4 ($p<0.001$, $R^2=0.69$). It is suspected that the choice of TSH for stimulation will be critical for success with TSH stimulations in odontocetes and it is likely that commercially available forms may not provide a strong secretagoge due to high levels of species variability in the structure of this hormone.

IMPACT/APPLICATIONS

The ability to identify stress markers relative to monitoring the health of marine mammal populations is critical to understanding the impact of anthropogenic activities upon those populations. The baseline characterization of stress marker variation in dolphins as a function of seasonality, gender, age, and reproductive status is important to assessing measurements made in wild dolphins. Information on

levels and dynamics of stress markers between different matrices provide better estimates of the overall condition of marine mammals sampled in the wild from either blubber biopsies or fecal collections. Sampling from these matrices may be the only means by which handling artifacts can be avoided in cetaceans. Understanding the function and dynamics of the HPA and HPT axes provides fundamental information on the stress response in these marine mammals, which may differ significantly from that of the terrestrial mammals from which most of our understanding is based. The incidental finding of the impact of MegAce on the dolphin endocrine system has broad-scale implications for the welfare of dolphins under human care. (Note*-Results are currently in the process of being published. A total of 6-7 pubications are expected from this work.)

RELATED PROJECTS

Project: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins (PI Pat Fair)
This project looks at numerous markers of stress in a wild population of marine mammals and compares them to animals under managed care in order to quantify and qualify the impact of environmental stressors on wild dolphins. The dolphins under managed care are from the Georgia Aquarium and the Navy Marine Mammal Program. Ten of the dolphins used in Task 1 of the current study (PI – Houser) were used as the semi-domesticated comparison.

Project: Validating the Novel Method of Measuring Cortisol Levels in Cetacean Skin by Use of an ACTH Challenge in Bottlenose Dolphins (PI Thea Bechsøft)
This project looks to characterize cortisol in the sloughed skin of odontocete cetaceans. Five dolphins used in the stress test of the current study (PI – Houser) were used for the sloughed skin collections.

Project: Quantifying stress in Marine Mammals: measuring biologically active cortisol in cetaceans and pinnipeds (PI Rudy Boonstra)
This project looks to characterize the equilibrium dissociation constant for CBG as well as the CBG binding capacity of plasma for a number of marine mammal species.

Project: Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population (expansion) (PI Dorian Houser)
This project provides funds supporting the statistical analysis and write-up of research results obtained under the parent project.

Project: Stress hormones and their regulation in a captive dolphin population (PI Cory Champagne)
This project increases the analysis from the "stress test" and TSH stimulation of the parent project by incorporating metabolomics analyses and measuring the additional compounds of reverse-T3 and corticosteroid binding protein.

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PUBLICATIONS

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